

A vaccine composition comprising an Immnoadjuvant  
compound consisting of a Rho GTPase family activator

**Field of the invention**

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The present invention relates to a vaccine composition comprising an immuno adjuvant compound, wherein said immuno adjuvant compound consists of a RHO GTPase family activator.

10 **Background of the invention**

Vaccines have proven to be successful, highly acceptable methods for the prevention of infectious diseases. There are cost effective, and do not induce antibiotic resistance to the target pathogen or affect normal flora present in the host. In many cases, such as when  
15 inducing anti-viral immunity, vaccines can prevent a disease for which there are no viable curative or ameliorative treatments available.

Vaccines function by triggering the immune system to induce a response to an agent, or an antigen, typically in an infectious organism or a portion thereof that is introduced into the body in a non-infectious or  
20 non-pathogenic form.

Once the immune system has been "primed" or sensitised to the organism, later exposure of the immune system to this organism, results in a rapid and robust immune response that destroys the pathogen before it can multiply or infect enough cells in the host organism to cause  
25 disease symptoms.

The agent, or antigen, used to prime the immune system can be the entire organism in a less infectious state, known as an attenuated organism, or in some cases, component of the organism such as carbohydrate proteins or peptides representing various structural  
30 components of the organism.

In many cases, it is necessary to enhance the immune response to the antigens present in a vaccine in order to stimulate the immune system to a sufficient extent to make a vaccine effective, i.e., to confer immunity. Many proteins and most peptide and carbohydrate antigens,  
35 administered alone, do not elicit a sufficient antibody response to confer

immunity. Such antigens need to be presented to the immune system in such a way that they will be recognized as foreign and will elicit an immune response.

To this end, additives like adjuvants, have been devised, which  
5 immobilise antigens and stimulate the immune response.

Recombinant proteins are promising vaccine or immunogenic composition candidates because they can be produced at high yield and purity and manipulated to maximize desirable activities and minimize undesirable ones.

10 However, because they can be poorly immunogenic, methods to enhance the immune response to recombinant proteins are important in the development of vaccines or immunogenic compositions. Such antigens, especially when recombinantly produced, may elicit a stronger response when administrated in conjunction with an adjuvant.

15 The best known adjuvant, Freund's complete adjuvant, consists of a mixture of mycobacteria in an oil/water emulsion.

Freund's adjuvant works in two ways; first, by enhancing cell and humoral-mediated immunity, and second by blocking rapid dispersal of the antigens challenge, also called "depot effect". However, due to  
20 frequent toxic physiological and immunological reactions to this material, Freund's adjuvant cannot be used in humans.

Another molecule that has been shown to have stimulatory or adjuvant activity is endotoxin, although known as lipopolysaccharide (LPS).

25 LPS stimulates the immune system by triggering an immediate immune response, a response that has evolved to enable an organism to recognize endotoxin and the invading bacteria (of which it is a component) without the need for the organism to have been previously exposed. But LPS is although too toxic to be a viable adjuvant.

30 Thus, there is a recognized and permanent need in the art for new compounds which can be administered with antigens in order to stimulate the immune system and generate a more robust antibody response to the antigen than will be seen if the antigens were injected alone.

Additionally, it should be noted that parenteral administration i.e. intramuscularly or sub-cutaneous, of antigens of vaccines are normally regarded as the most convenient way of administration.

However, the injection presents a range of disadvantages. It requires the use of sterile syringes and may cause pains and irritations, particularly in the case of repeated injections, including the risk of infection. More significantly, intramuscularly injections are often poorly tolerated. There is often likely to be indurations (hardening of tissue) haemorrhages and/or necrosis (local death of tissue) at the injection site. Besides, untrained person cannot administer injections.

Based on these observations, it should be noted that mucosal immunity has take a considerable importance in vaccine development because nearly all viral, bacterial and parasitic agent that cause disease of the intestinal, respiratory and genital tracks enter through the mucosal barrier. Furthermore, mucosal and systemic immune responses are often elicited and regulated independently, and induction of protective immunity at the most frequent sites of entry is likely to be most effective. Additionally, young children and elderly individuals may respond better to mucosal vaccines because the mucosal immune system develops earlier and appears to remain functional longer than the systemic compartment. Mucosal immunisations are also easier and less expensive than systemic immunisations. For example, the existence of an oral polio vaccine has allowed immunisation campaigns that may soon eradicate polio worldwide.

Accordingly, it is also an object of the present invention to provide a vaccine composition comprising an immunoadjuvant compound which could be administered by the mucosal route. These and further objects will be apparent to one ordinary skill in the art.

### **Summary of the invention**

The present invention is based on the experimental findings that an activator of Rho GTPases, namely the cytotoxic necrotizing factor 1 (cnf1) bears immunostimulatory properties towards the systemic and mucosal responses to orally administered ovalbumine, a prototype soluble protein antigen. CNF1 consists of an injection domain (amino

acid residues 1-719 of SEQ ID N°1), allowing the binding and endosomal penetration of the toxin, followed by the intracytoplasmic injection of its catalytic domain (amino acid residues 720-1014 of SEQ ID N°1), responsible for Rho GTPases protein family activation.

5 A first object of the invention consists in a vaccine composition comprising an immunoadjuvant compound, wherein said immunoadjuvant compound consists of a Rho GTPase activator.

In another aspect, the invention relates to a vaccine composition wherein said immunoadjuvant compound is selected from the group  
10 consisting of :

- a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°1,
- a polypeptide comprising the amino acid sequence starting at the  
15 amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°2,
- a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°3,
- 20 - a polypeptide comprising the amino acid sequence starting at the amino acid residue 1146 and ending at the amino acid residue 1451 of sequence SEQ ID N°4,
- a polypeptide comprising the amino acid sequence SEQ ID N°5,
- a polypeptide comprising the amino acid sequence SEQ ID N°6,
- 25 - a polypeptide comprising the amino acid sequence SEQ ID N°7,
- a polypeptide comprising the amino acid sequence SEQ ID N°8, and
- a polypeptide comprising the amino acid sequence SEQ ID N°9.

The present invention also relates to a vaccine composition wherein the immunoadjuvant compound is a protein comprising a polypeptide  
30 consisting of; from the N-terminal end to the C-terminal end, respectively:

- a) the injection domain of a Rho GTPase activator , and
- b) the catalytic domain of a Rho GTPase activator.

### **Description of drawings**

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**Figure 1 : CNF1 effects on cell signaling pathway.**

- 1A : Immunoblots showing the kinetics of CNF1-induced activation of Rho, Rac and Cdc42 in contrast to Ras, in HUVEC. Cells were treated with  $10^{-9}$ M CNF1 for different periods of time. Cell lysates were subjected to GST-fusion protein pull-down assays (noted GTPases-GTP). In parallel, 2% of each cell lysate were processed for immunoblotting to monitor their cellular depletion (noted Total-GTPases).
- 1B : Quantification of the CNF1-induced Rho protein activation. Immunoblots were scanned and quantified using N.I.H. Image 1.6. The level of activated Rho proteins was compared to the total Rho GTPase level present in 2% of control cell lysates (mean value of three independent experiments  $\pm$  SD).
- 1C : Immunoblots showing the interference of native CNF1 and catalytic inactive CNF1-C866S on cell signaling. HUVEC were treated with " $10^{-9}$ M" CNF1 or CNF1-C866S for the indicated periods of time, prior to immunoblotting analysis. MAP kinase signaling was investigated using anti-phosphop44/42 MAP Kinase (noted P-p44/42) and anti-phospho-p38 MAP Kinase (noted P-p38) antibodies. Jun kinase activity was investigated by anti-phospho-c-jun (noted P-c-jun) immunoblotting. NF-kappaB signaling pathway activation was investigated by following the I $\kappa$ B $\alpha$  cellular depletion on immunoblots.

**Figure 2 : Catalytic active CNF1 stimulates serum IgG responses to orally administered ovalbumin (OVA).**

- Five groups of mice were fed OVA alone (control) or co-administered with either CNF1 (1 or 10  $\mu$ g) or CNF1-C866S (10  $\mu$ g) or CT (10  $\mu$ g). Groups of eight mice were immunized with CNF1 or CNF1-C866S, whereas groups of four mice were immunized with OVA alone or OVA+CT. Groups of mice were challenged once, 2 weeks after the first immunization and sera collected 30 days after the first immunization. Levels of the seric anti-OVA IgG titers are expressed as geometric

means (histogram and mean values) of the total IgG titers. These results are representative of two independent experiments. Anti-OVA IgG titers from individual animals are displayed (•).

5    **Figure 3 DNT catalytic domain stimulates serum IgG responses to orally administered ovalbumin (OVA).**

10    - 3A : Immunoblots showing the kinetics of DNT-Cdinduced activation and cellular depletion of Rac. 804G cells were treated with 100 µg of DNT-CD and processed for activated Rac measurements by GST-Pak pull-down (noted RacGTP). Immunoblotting of 10 µg of total lysate was performed to visualize DNT-CD induced Rac depletion (noted Rac) and equal quantities of proteins engaged in the GST pull-down (actin).

15    - 3B : Comparison of the cellular activities of CNF-CD and DNT-CD. The graph illustrates the percentage of HEp-2 multinucleated cells measured 48 h following intoxication by different concentrations of either CNF-CD or DNT-CD.

20    - 3C : Serum IgG responses to orally administered ovalbumin (OVA). Three groups of 4 mice were fed with OVA alone (control) or co-administered with either CNF-CD (100 µg) or DNT-CD (100 µg). Groups of mice were challenged twice, 2 and 5 weeks after the first immunization and sera collected 30 and 60 days after the first immunization. Levels of the seric anti-OVA IgG titers are expressed as geometric means of the  
25    total IgG titers.

**Figure 4 CNF1, CNF1-C866S and CT induction of anti-OVA Ig subclasses.**

30    Three groups of three mice were challenged twice after the first immunization and sera collected 45 days after the first immunization. Levels of the anti-OVA Ig subclasses are expressed as geometric means (histogram).

35    **Figure 5 : CNF1 induction of mucosal anti-OVA IgA response.**

Two groups of three mice were challenged twice, after the first immunization, with OVA supplemented with 10 µg of either CNF1 or CNF1-C866S. Mice were processed according to the PERFEXT method (see the section Material and Method). Levels of the anti-OVA IgA responses are expressed as geometric means (histogram).

**Figure 6 : Histology of small intestines of mice fed CNF1 or CNF1-C866S as compared to control untreated mice.**

Shown are paraffin sections stained with haematoxylin and eosin.

**Figure 7 : Measure of the immunoadjuvant properties and toxin activity of CNF1 and DNT.**

- 7A: Measure of the toxin activity of CNF1, CNF1-CTER (720-1014), DNT-CTER (1154-1451) estimated by HEp-2 cells multinucleation assay, as previously described (Lemichez et al., 1997). As previously reported, CNF1-CTER is poorly active on cells due to its inability to penetrate into the cytosol (Lemichez et al., 1997). DNT-CTER shows a one thousand lower activity, as compared to CNF1.
- 7B: Serum IgG antibody responses to orally co-administered ovalbumin (OVA) and DNT or CNF1-toxin catalytic domains. Groups of 4 mice were fed OVA alone or co-administered with either CNF1-CTER (720-1014) (100µg) or DNT-CTER (1154-1451) (100µg). For CNF1, a group of eight mice were fed OVA and CNF1 (10µg). Mice were challenged once, two weeks after the first immunization and sera collected 30 days after the first immunization. Data are expressed as geometric mean serum IgG anti-OVA Ab titers.

**DETAILED DESCRIPTION OF THE INVENTION**

The inventors have found according to the invention that Rho GTPase activators bear immunoadjuvant properties *in vivo*, when co-administered with an antigen, like ovalbumin.

Rho proteins are essential regulatory molecules controlling the actin cytoskeleton organisation and dynamics to accomplish different tasks such as cell polarity, movement, differentiation and phagocytosis (Takai et al., 2001, Etienne-Manneville et al., 2002, Chimini and Chavrier, (2000)). Importance of Rho proteins in physiology is also evidenced by their direct or indirect implication as part of signaling molecules found mutated in human genetic disorders, as well as targets of numerous bacterial virulence factors and toxins (Boettner and Van Aelst, (2002) Boquet and Lemichez, (2003).

Rho proteins interfere with a large variety of signaling pathways controlling gene transcription (Bishop et al., 2000). Among them, a recent report has evidenced the activation of Rac and Cdc42 downstream the Toll-like receptor 2, a gram positive pathogen molecular pattern recognition receptor (PAMP) (Arbibe et al. (2000), Medzhitov et al. (2002).

Also exemplifying the inter-relation between Rho proteins and the host defences is the Rac, Cdc42, VAV and WASP formation of a supra-molecular activation complex (SMAC or "immunological synapse" crucial for lymphocyte activation (Krawczyk et al. 2001).

Many different pathogenic bacteria have evolved virulence factors and toxins aimed at mimicking an activation of Rho GTPase protein family, naturally occurring in eukaryotic cells via specific regulators namely GEF (for guanine nucleotide exchange factors). These cellular GEF consist in domains comprised in large proteins as best described for DbI (Olson et al., 1996; Schmidt and Hall 2002). Despite their lack of sequence homologies, virulence factors of pathogenic bacteria, for instance SopE and SopE2 from *Salmonella* have a GEF-like activity (Galan et al., 2000). Some other known factors of pathogenic bacteria, namely IpaC from *Shigella* and CagA from *Helicobacter*, activate Rho GTPases by yet uncharacterised molecular mechanisms (Tran Van Nhieu et al., 2000; Boquet and Lemichez 2003). Finally, a group of bacterial toxins comprising CNF1 also activates Rho proteins through a post-traductional modification (Boquet and Lemichez 2003)

According to the invention, the inventors have now surprisingly found that the cytotoxic necrotising factor 1 (CNF1), has immunoadjuvant



properties. More precisely, the inventors have found that CNF1 bears immunostimulatory properties toward the systemic and mucosal responses to orally administrated ovalbumin in mice.

Additionally, the inventors have found that a mutant of CNF1, namely CNF1-C866S, a catalytically inactive mutant of CNF1 toward GTPases, in contrast to the wild type toxin, does not stimulate the systemic and mucosal responses to ovalbumin. This result points for Rho GTPases proteins activation being directly involved in the immunostimulatory effects of CNF1.

Supporting this point, the inventors have also found according to the invention that the catalytic domain of CNF1, and the catalytic domain of DNT, another Rho GTPase activator, bear also immunoadjuvant properties *in vivo*, when co-administered with an antigen, like ovalbumin.

Taken together, these results demonstrate clearly that different Rho GTPases activators, structurally different, have immunoadjuvant properties.

Furthermore, the inventors have found that non neutralizing anti-CNF1 antibodies are naturally found in humans, and that CNF1 activates the Rho GTPase proteins only transiently. Taken together these results demonstrate that CNF1 can be used as an immunoadjuvant compound, deserved of adverse effects such as the toxic effects described for LPS or Cholera Toxin B.

Accordingly, a first object of the invention consists in a vaccine composition comprising an immunoadjuvant compound, wherein said immunoadjuvant compound consists of a Rho GTPase activator.

By "immunoadjuvant" it is herein intended a substance enhancing the immunogenicity of an antigen. By "Rho GTPase activator" it is intended herein a compound, which maintains Rho GTPases in a form bound to GTP. By "Rho GTPases", the one skilled in the art will understand the proteins belonging to the Rho GTPase family, which encompasses RhoA, RhoB, RhoC, Rac1, Rac2 and Cdc42. (Burridge and Wennerberg, 2004).

The level of Rho GTPase bound to GTP can be easily measured by the methods, referred by those skilled in the art as GST-pull down

assays and described for RhoA, B and C by Ren et al., 1999 and for Rac1, Rac2 and Cdc42 by Manser et al., 1998. These methods are described in the section Materials and methods.

The invention also concerns a vaccine composition as described below, wherein said immunoadjuvant is selected from the group consisting of :

- a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°1,
- 10 - a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°2,
- a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°3,
- 15 - a polypeptide comprising the amino acid sequence starting at the amino acid residue 1146 and ending at the amino acid residue 1451 of sequence SEQ ID N°4,
- a polypeptide comprising the amino acid sequence SEQ ID N°5,
- 20 - a polypeptide comprising the amino acid sequence SEQ ID N°6,
- a polypeptide comprising the amino acid sequence SEQ ID N°7,
- a polypeptide comprising the amino acid sequence SEQ ID N°8, and
- a polypeptide comprising the amino acid sequence SEQ ID N°9.

A Rho GTPase activator encompasses peptides comprising the amino acid sequence of interest starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°1 described above, and comprising a N-terminal amino acid sequence, linked to the amino group of the residue 720 of sequence SEQ ID N°1.

Preferably, the N-terminal amino acid sequence has a length up to 800 amino acid residues.

Preferably, the N-terminal amino acid sequence is homologous to a part or to the full length amino acid sequence starting at the amino acid residue 1 and ending at the amino acid residue 719 of CNF1 of SEQ ID N°1.

In such a case, the N-terminal amino acid sequence can comprise substitutions of non-essential amino acid comprised in the sequence starting at the amino acid residue 1 and ending at the amino acid residue 719 of CNF1 of SEQ ID N°1.

5 A "non essential" amino acid residue is an amino acid residue that can be altered from the wild type sequence of CNF1 without altering the activating properties of Rho GTPases, whereas an "essential" amino acid residue is required for biological activity.

10 A Rho GTPase activator encompasses also peptides comprising two or more repeated motifs of the sequence 720-1014 of interest. In such a case, said peptide can comprise also an N-Terminal sequence as defined above.

15 A Rho GTPase activator encompasses also peptides structurally similar to those described above, derived from the catalytic domain of CNF2 of sequence SEQ ID N°2, the catalytic domain of CNF $\gamma$  of sequence SEQ ID N°3 and the catalytic domain of DNT of sequence SEQ ID N°4.

20 The use of the catalytic domain of Rho GTPase activator, as described above, is of particular interest. Indeed, as demonstrated in example 6, in the case of CNF1, and DNT, the use of the catalytic domain of these proteins is less toxic for cells than the overall proteins, but is sufficient to confer immunoadjuvanticity.

A Rho GTPase activator encompasses also peptides comprising :

- the amino acid sequence SEQ ID N°5 corresponding to SOPE, or
- 25 - the amino acid sequence SEQ ID N°6 corresponding to SOPE2, or
- The amino acid sequence SEQ ID N°7 corresponding to IpaC, or
- the amino acid sequence SEQ ID N°8 corresponding to CagA, or
- the amino acid sequence SEQ ID N°9 corresponding to the GEF sequence of DbI,
- 30 which include more amino acids, and exhibit at least the same activity towards Rho GTPase activation.

Alternatively, the immunoadjuvant according to the invention is selected from the group consisting of :

35 - a polypeptide comprising the amino acid sequence SEQ ID N°1,  
- a polypeptide comprising the amino acid sequence SEQ ID N°2,

- a polypeptide comprising the amino acid sequence SEQ ID N°3, and
- a polypeptide comprising the amino acid sequence SEQ ID N°4.

Another object of the invention consists in a vaccine composition, wherein said immunoadjuvant compound is a protein comprising a polypeptide consisting of; from the N-terminal end to the C-terminal end,  
5 respectively:

- a) the injection domain of a Rho GTPase activator , and
- b) the catalytic domain of a Rho GTPase activator.

By "injection domain of a Rho GTPase activator" it is intended  
10 herein, an amino acid sequence allowing the binding and intracellular penetration of a catalytic domain of a Rho GTPase activator.

By "catalytic domain of a Rho GTPase activator" it is intended herein, an amino acid sequence able to activate a Rho GTPase.

The attachment of the injection domain to the catalytic domain  
15 above mentioned, to produce a fusion protein may be effected by any means which produces a link between the two constituents, which is sufficiently stable to withstand the conditions used and which does not alter the function of either constituent.

Preferably, the link between them is covalent.

20 Numerous chemical cross-linking methods are known and potentially applicable for producing the fusion protein. For example, non-specific chemical cross-linking methods, or preferably methods of direct chemical coupling to a functional group, found only once or a few times in one or both of the polypeptides to be cross-linked.

25 Coupling of the two constituents can also be accomplished via a coupling or conjugating agent. There are several intermolecular cross-linking reagents, which can be used (see, for example, Means, G. E. et al. (1974)). Among these reagents are, for example, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) or N, N'-(1,3-phenylene) bismaleimide.

30 Cross-linking reagents may be homobifunctional, i.e., having two functional groups that undergo the same reaction such as bismaleimido-hexane ("BMH").

Alternatively, to solve the problems of protein denaturation and contamination during chemical conjugation, recombinant techniques can  
35 be used to covalently attach the polypeptide of interest to the virulence

factor, such as by joining the nucleic acid coding for the polypeptide of interest with the nucleic acid sequence coding for the virulence factor and introducing the resulting gene construct into a cell capable of expressing the conjugate.

5           Recombinant methodologies required to produce a DNA encoding a desired protein are well known and routinely practiced in the art. Laboratory manuals, for example MOLECULAR CLONING: A LABORATORY MANUAL. Cold Spring Harbor Press: Cold Spring Harbor, N.Y. (1989) describes in detail techniques necessary to carry out  
10       the required DNA manipulations.

          The fusion protein can be produced in recombinant microorganism transformed therewith. In this process, each protein component is preferably linked in the molecular ratio of 1:1 (injection domain : catalytic domain). The aid of an appropriate linker, in order to allow proper folding  
15       of each protein molecule can be useful. As a linker, it is preferable to use a peptide consisting of the appropriate number of amino acids to maintain activity of each protein component, such as, a peptide composed of 0 to 20 amino acids, though glycine, (glycine)<sub>4</sub> serine, or [(glycine)<sub>4</sub> serine]<sub>2</sub>.

20           Preferable vectors include any of the well known prokaryotic expression vectors, recombinant baculoviruses, COS cell specific vectors, or yeast-specific expression constructs.

          Alternatively, the two separate nucleotide sequences can be expressed in a cell or can be synthesized chemically and subsequently  
25       joined, using known techniques. Alternatively, the fusion protein can be synthesized chemically as a single amino acid sequence (i.e., one in which both constituents are present) and, thus, joining is not needed.

          Preferably, the injection domain of a Rho GTPase activator is a polypeptide selected from the group consisting of :

- 30       - a polypeptide comprising the amino acid sequence starting at the amino acid residue 1 and ending at the amino acid residue 719 of sequence SEQ ID N°1;
- a polypeptide comprising the amino acid sequence starting at the amino acid residue 1 and ending at the amino acid residue 719 of  
35       sequence SEQ ID N°2;

- a polypeptide comprising the amino acid sequence starting at the amino acid residue 1 and ending at the amino acid residue 719 of sequence SEQ ID N°3; and
- 5     - a polypeptide comprising the amino acid sequence starting at the amino acid residue 1 and ending at the amino acid residue 1145 of sequence SEQ ID N°4.

Preferably, the catalytic domain of a Rho GTPase activator is a polypeptide selected from the group consisting of :

- 10     - a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°1,
- a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°2,
- 15     - a polypeptide comprising the amino acid sequence starting at the amino acid residue 720 and ending at the amino acid residue 1014 of sequence SEQ ID N°3,
- a polypeptide comprising the amino acid sequence starting at the amino acid residue 1146 and ending at the amino acid residue 1451 of sequence SEQ ID N°4,
- 20     - a polypeptide comprising the amino acid sequence SEQ ID N°5,
- a polypeptide comprising the amino acid sequence SEQ ID N°6,
- a polypeptide comprising the amino acid sequence SEQ ID N°7,
- a polypeptide comprising the amino acid sequence SEQ ID N°8, and
- 25     a polypeptide comprising the amino acid sequence SEQ ID N°9.

The invention concerns also the vaccine composition as described above, further comprising an antigen.

Preferably, the antigen is selected from the group consisting of a hormone, a protein, a drug, an enzyme, a vaccine composition against  
30     bacterial, viral, fungal, prion, or parasitic infections, a component produced by microorganisms, inactivated bacterial toxins such as cholera toxin, ST and LT from *Escherichia coli*, tetanus toxin from *Clostridium tetani*, and proteins derived from HIV viruses.

The amount of antigen, and immunoadjuvant compound in the  
35     vaccine composition according to the invention, the dosages

administered, are determined by techniques well known to those skilled in the pharmaceutical art, taking into consideration such factors as the particular antigen, the age, sex, weight, species, and condition of the particular animal or patient, and the route of administration.

5           In a preferred embodiment, the vaccine composition according to the invention, further comprises one or more components selected from the group consisting of surfactants, absorption promoters, water absorbing polymers, substances which inhibit enzymatic degradation, alcohols, organic solvents, oils, pH controlling agents, preservatives,  
10       osmotic pressure controlling agents, propellants, water and mixture thereof.

          The vaccine composition according to the invention can further comprise a pharmaceutically acceptable carrier. The amount of the carrier will depend upon the amounts selected for the other ingredients,  
15       the desired concentration of the antigen, the selection of the administration route, oral or parenteral, etc. The carrier can be added to the vaccine at any convenient time. In the case of a lyophilised vaccine, the carrier can, for example, be added immediately prior to administration. Alternatively, the final product can be manufactured with  
20       the carrier.

          Examples of appropriate carriers include, but are not limited to, sterile water, saline, buffers, phosphate-buffered saline, buffered sodium chloride, vegetable oils, Minimum Essential Medium (MEM), MEM with HEPES buffer, etc.

25           Optionally, the vaccine composition of the invention may contain conventional, secondary adjuvants in varying amounts depending on the adjuvant and the desired result. The customary amount ranges from about 0.02% to about 20% by weight, depending upon the other ingredients and desired effect.

30           Examples of suitable secondary adjuvants include, but are not limited to, stabilizers; emulsifiers; aluminum hydroxide; aluminum phosphate; pH adjusters such as sodium hydroxide, hydrochloric acid, etc.; surfactants such as Tween.RTM. 80 (polysorbate 80, commercially available from Sigma Chemical Co., St. Louis, Mo.); liposomes; iscom  
35       adjuvant; synthetic glycopeptides such as muramyl dipeptides; extenders

such as dextran or dextran combinations, for example, with aluminum phosphate; carboxypolymethylene; bacterial cell walls such as mycobacterial cell wall extract; their derivatives such as *Corynebacterium parvum*; *Propionibacterium acne*; *Mycobacterium bovis*, for example, 5 *Bovine Calmette Guerin* (BCG); vaccinia or animal poxvirus proteins; subviral particle adjuvants such as orbivirus; cholera toxin; N,N-di-octadecyl-N',N'-bis(2-hydroxyethyl)-propanediamine (avridine); monophosphoryl lipid A; dimethyldioctadecylammonium bromide (DDA, commercially available from Kodak, Rochester, N.Y.); synthetics and 10 mixtures thereof. Desirably, aluminum hydroxide is admixed with other secondary adjuvants or an immunoadjuvant such as Quil A.

Examples of suitable stabilizers include, but are not limited to, sucrose, gelatin, peptone, digested protein extracts such as NZ-Amine or NZ-Amine AS. Examples of emulsifiers include, but are not limited to, 15 mineral oil, vegetable oil, peanut oil and other standard, metabolizable, nontoxic oils useful for injectables or intranasal vaccines compositions.

For the purpose of this invention, these adjuvants are identified herein as "secondary" merely to contrast with the above-described immunoadjuvant compound, consisting of a Rho GTPase activator, that 20 is an essential ingredient in the vaccine composition for its effect in combination with an antigenic substance to significantly increase the humoral immune response to the antigenic substance. The secondary adjuvants are primarily included in the vaccine formulation as processing aids although certain adjuvants do possess immunologically enhancing 25 properties to some extent and have a dual purpose.

Conventional preservatives can be added to the vaccine composition in effective amounts ranging from about 0.0001% to about 0.1% by weight. Depending on the preservative employed in the formulation, amounts below or above this range may be useful. Typical 30 preservatives include, for example, potassium sorbate, sodium metabisulfite, phenol, methyl paraben, propyl paraben, thimerosal, etc.

The choice of inactivated, modified or other type of vaccine composition and method of preparation of the improved vaccine composition formulation of the present invention are known or readily 35 determined by those of ordinary skill in the art.



A pharmacologically effective amount of the immunoadjuvant compound according to the invention may be given, for example orally, parenterally or otherwise, concurrently with, sequentially to or shortly after the administration of a an antigenic substance in order to potentiate,  
5 accelerate or extend the immunogenicity of the antigen.

While the dosage of the vaccine composition depends upon the antigen, species, body weight of the host vaccinated or to be vaccinated, etc., the dosage of a pharmacologically effective amount of the vaccine composition will usually range from about 50 µg to about 500 µg per  
10 dose, per kilogram of body weight, in a mouse model.

Although the amount of the particular antigenic substance in the combination will influence the amount of the immunoadjuvant compound according to the invention, necessary to improve the immune response, it is contemplated that the practitioner can easily adjust the effective  
15 dosage amount of the immunoadjuvant compound through routine tests to meet the particular circumstances.

As a general rule, the vaccine composition of the present invention is conveniently administered orally, parenterally (subcutaneously, intramuscularly, intravenously, intradermally or intraperitoneally),  
20 intrabuccally, intranasally, or transdermally. The route of administration contemplated by the present invention will depend upon the antigenic substance and the co-formulants. For instance, if the vaccine composition contains saponins, while non-toxic orally or intranasally, care must be taken not to inject the sapogenin glycosides into the blood  
25 stream as they function as strong hemolytics. Also, many antigens will not be effective if taken orally. Preferably, the vaccine composition is administered subcutaneously, intramuscularly or intranasally.

The dosage of the vaccine composition will be dependent upon the selected antigen, the route of administration, species, body weight  
30 and other standard factors. It is contemplated that a person of ordinary skill in the art can easily and readily titrate the appropriate dosage for an immunogenic response for each antigen to achieve the effective immunizing amount and method of administration.

The inventors have also shown, in example 1 that CNF1 has  
35 Immunoadjuvant properties when coadministered orally with an antigen.

They have also shown that this coadministration enhances the total IgA antibody titer in mice. This last result is typical of a mucosal response to an immunisation.

Consequently, a further object of the invention is a vaccine composition according to the invention, for administration to a mucosal surface.

This mode of administration presents a great interest. Indeed, the mucosal membranes contain numerous of dendritic cells and Langerhans cells, which are excellent antigen detecting and antigen presenting cells. The mucosal membranes are also connected to lymphoid organs called mucosal associated lymphoid tissue, which are able to forward an immune response to other mucosal areas. An example of such an epithelia is the nasal epithelial membrane, which consists of practically a single layer of epithelial cells (pseudostratified epithelium) and the mucosal membrane in the upper respiratory tract is connected to the two lymphoid tissues, the adenoids and the tonsils. The extensive network of blood capillaries under the nasal mucosal of the high density of B and T cells, are particularly suited to provide a rapid recognition of the antigen and provide a quick immunological response.

Preferably, the mucosal surface is selected from the group consisting of mucosal surfaces of the nose, lungs, mouth, eye, ear, gastrointestinal tract, genital tract, vagina, rectum, and the skin.

Another object of the invention is a vaccine composition for an oral administration.

The invention concerns also a protein comprising a polypeptide consisting of; from the N-terminal end to the C-terminal end, respectively:

- a) the injection domain of a Rho GTPase activator as described above, and
- b) the catalytic domain of a Rho GTPase activator as described above.

The invention further concerns the use of a polypeptide of interest, for manufacturing a vaccine composition.

The invention also concerns the use of a fusion protein as described above for manufacturing a vaccine composition.

Further details of the invention are illustrated in the following non-limiting examples.

## **MATERIALS AND METHODS**

### **Cells and reagents**

Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell (Heidelberg, Germany). Cells were grown in Human Endothelial SFM medium (Invitrogen Co, Paisley, Scotland) supplemented with defined growth factors (d-SFM): 10 ng/ml EGF and 20 ng/ml bFGF (Invitrogen Co), 1 µg/ml heparin (Sigma-Aldrich) and either 20% foetal bovine serum (Invitrogen Co) or 1% (W/V) bovine serum albumin (ELISA grade, Sigma-Aldrich) together with penicillin and streptomycin (Invitrogen Co). Cells were grown on 0,2% gelatine coated dishes (Sigma-Aldrich). Transfections of HUVEC were carried out as described by Mettouchi et al., 2001. Antibodies used were monoclonal anti-β actin antibody [clone AC-74] (Sigma-Aldrich); anti-RhoA, anti-Cdc42, anti-Rac1 and anti-Ras antibodies (Transduction Laboratories); anti-HA [clone 11] (BabCO); anti-E-selectin [clone CTB202] (Santa Cruz Biotechnology) and rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti phospho-p38 MAP kinase (Thr180/Tyr182) and anti phospho-c-Jun (Ser73) (Cell Signaling Technology); anti-human IκB-α (Upstate Biotechnology); anti-TRAF1 (H-186, Santa Cruz Biotechnology). Primary antibodies were visualized using goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). TRAF1 rabbit antibodies were visualized using biotin-XX goat anti-rabbit IgG followed by streptavidin horseradish peroxidase conjugate (Molecular Probes). DNA vectors corresponding to pcDNA3RhoQ63L, RacQ61L and Cdc42Q61L were provided by Manor, D. ( Lin et al., 1999).

### **Toxins**

Purified CT was obtained from List Biologicals (Campbell, CA). CNF1 and CNF1-C866S toxins production and purification were performed as previously described (Munro et al., 2004). Briefly, overnight cultures of E. coli OneShot, carrying pCR2cnf1 or pCR2cnf1C866S were lysed in PBS using a French Press. After ammonium sulfate precipitation and dialysis

against Tris–NaCl buffer, the soluble fraction was then applied to series of column purifications. Protein purification was followed by SDS-PAGE. The activity of the different batches of CNF1 toxin was estimated by multinucleation assay, as previously described Lemichez et al., 1997).

5 The purified CNF1 toxin used in this study produced, at 10–12 M, 50% of multinucleation of HEp-2 cells after 48 h of exposure. CNF1 catalytic domain (amino acids 720–1014) and DNT catalytic domain (amino acids 1154–1451) were produced using the same methods and activities were assessed as described earlier for CNF1. All protein preparations were

10 found to contain doses of endotoxin below 0.12 EU/ml of FDA Reference Standard, using the Multi-Test Limulus Amebocyte Lysate Pyrogen Plus® (Biowhittaker, Walkersville, MD). Activation and degradation of Rac was assessed using GST-protein pulldown experiment as previously described (Doye et al., 2002).

15

### **Immunizations**

Females BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France). They were maintained and handled according to the regulations of the European Union and the French Department of Health.

20 In all experiments, 4–8 week-old female mice were used. Mice were fed either CNF1, CNF1-C866S (a catalytic inactive toxin), catalytic domains of CNF1 (CNF-CD) and DNT (DNT-CD) or CT in the presence or absence of 5mg of ovalbumin (OVA) (grade V, Sigma–Aldrich, St. Louis, MO) dissolved in a solution of 500µl of 3% NaHCO<sub>3</sub>. Animals were fed

25 on either two or three consecutive occasions, as detailed in figure legends, 10–12 days apart.

### **Measurements of serum antibody responses**

Serum antibody levels against OVA were determined by means of solid-phase ELISA, as previously described (anjuère et al., 2003). Briefly,

30 serial three-fold dilutions of test and control sera were incubated for 2 h at room temperature in OVA-coated polystyrene microtiter wells (Nunc-Immuno™ Plates, MaxiSorp™ Surface, Nunc, Denmark). After washes with PBS containing 0.05% Tween, wells were exposed to 0.1 ml of

35 PBS–Tween containing appropriately diluted HRP-conjugated goat anti-

mouse IgG, IgG1, IgG2a, IgG2b and IgA (Southern Biotech Inc., Birmingham, AL). Plates were developed with BM blue, POD chromogenic substrate (Roche Applied Science, Indianapolis, IN) and monitored spectrophotometrically. Titers were defined as the reciprocal of the highest dilution of serum giving an absorbance value of twice above control, corresponding to pre-immune serum.

#### **Measurements of mucosal antibody responses**

Six days after the last immunization, mice were anesthetized with entobarbital and injected with pyrogen-free isotonic saline containing 100 units heparin. The carotid vein was cut and animals were perfused in situ with 25 ml of PBS containing 100 units/ml heparin administered by intracardiac injection to minimize contamination with blood. The small intestines were resected, opened longitudinally and washed with PBS. Sections were cut into small fragments and further perfused with PBS—heparin for 4 h at 4 °C. Tissue fragments were weighed and then manipulated according to the PERFEXT method, based upon sequential perfusion and detergent extraction (Villavedra et al., 1997). Briefly, fragments were homogenized, suspended in 2ml of extraction buffer/mg of tissue and incubated overnight at 4 °C. The extraction buffer consisted of PBS supplemented with 2% saponin (Sigma) and protease inhibitors (Complete, Boehringer). Samples were then kept frozen at -80°C until assayed. Thirty minutes before use, specimens were allowed to thaw at room temperature and spun at 16,000×g for 10 min. Supernatants were assayed for IgA and IgG anti-OVA antibody titers as described earlier.

#### **Histology**

Mice were fed CNF1 or CNF1-C866S. After 48 h, mice were killed and the small intestines were collected, fixed in formalin and embedded in paraffin wax. Consecutive 5 µm paraffin sections were stained with haematoxylin and eosin.

#### **DNA array analysis**

HUVEC were seeded at  $8 \times 10^6$  cells/150 mm gelatin-coated dish in d-SFM containing BSA. Cells were intoxicated in parallel for 3h and 24h in d-

SFM/BSA supplemented with  $10^{-9}$ M CNF1. Cells were lysed in RTL buffer for total RNA extraction, according to the manufacturer (RNeasy MiniKit, Qiagen). CNF1 regulated genes were analyzed using Affymetrix® Human GeneChip U133A and U133B, by Aros Applied  
5 Biotechnology ApS (www.arosab.com), as recommended by the manufacturer (www.Affymetrix.com).

### **ELISA**

HUVEC were seeded 24h before toxin addition at  $2 \times 10^5$  cells/ 22.5 mm or  
10  $5 \times 10^5$  cells/ 35 mm well in d-SFM containing serum. Intoxication of cells was performed by addition of fresh medium containing CNF1, for different periods of time. One hour before intoxication ending the medium was replaced by d-SFM containing BSA for ELISA. IL-8, MCP-1, IL-6, MIP3- $\alpha$ , TNF- $\alpha$  and RANTES production were assessed using human  
15 Quantikine® immunoassays, as recommended by the manufacturer (R & D Systems, Abingdon, UK).

### **Pull-down and immunoblotting detection of activated-Rho GTPases**

Levels of activated-RhoA, -RhoB, -RhoC, -Rac1, -Rac2, -Cdc42 were  
20 measured using classical Rho effector pull-down assays developed by Manser et al., 1998 and Ren et al., 1999. For antibodies description see the cells and reagents section.

Briefly, the measure of the levels of activated-RhoA, -B and -C was performed as followed. Cells were lysed in 50mM Tris, pH7.2, 500mM  
25 NaCl, 10mM MgCl<sub>2</sub>, 1% Triton X-100, 0.5% deoxicholate, 0.1% SDS and protease inhibitors. Cell lysates were clarified by centrifugation at 13000g at 4°C for 10min. and equal volumes of lysates (corresponding to 1mg of total proteins) were incubated with 30 micrograms GST-RBD (Rho binding domain of Rhotekin fused to GST and described in Ren et al.,  
30 1999) beads at 4°C for 45min. The beads were washed four times with buffer B (50mM Tris, pH7.2, 500mM NaCl, 10mM MgCl<sub>2</sub>, 1% Triton X-100 and protease inhibitors). Bound Rho proteins were resolved by SDS-PAGE and transferred on PVDF membranes. Activated-Rho proteins were detected by immunoblotting using a monoclonal antibody against  
35 either RhoA and RhoC or RhoB and anti-mouse horseradish peroxidase-

conjugated secondary antibody followed by chemiluminescence detection.

The measure of the levels of activated-Rac1, Rac2 and Cdc42 was determined, as followed. Cells were lysed in LB buffer (25 mM Tris, pH7.5, 150mM NaCl, 5mM MgCl<sub>2</sub>, 0.5% Triton X-100, 4% glycerol and protease inhibitors). Cell lysates were clarified by centrifugation at 13000g at 4°C for 10min. and equal volumes of lysates (corresponding to 1mg of total proteins) were incubated with 30 micrograms GST-PAK70-106 (Rac/Cdc42 binding domain of p21PAK fused to GST and described in Manser et al., 1998) beads at 4°C for 45min. The beads were washed four times with LB. Bound Rac and Cdc42 proteins were resolved by SDS-PAGE and transferred on PVDF membranes. Activated-Rac1, 2 or activated-Cdc42 proteins were detected by immunoblotting using a monoclonal antibody against either Rac1, 2 or Cdc42 and anti-mouse horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence detection.

For activated Ras measurements GST-RBD1-149 of Raf1 was used as described by the authors (de Rooij and Bos, 1997).

#### 20 **Example 1: CNF1 effects on cell signaling pathways**

Kinetics of CNF1-induced Rac1, Cdc42 and RhoA activation have been studied. These kinetics show the specificity of Rho protein activation, as compared to the Ras GTPase (Fig. 1A, 1B). Obviously, these measurements do not represent an exhaustive list of the Rho proteins activated by CNF1, other Rho bearing the canonical sequence for CNF1 recognition/modification (Lerm et al., 1999). These measurements rather indicated that all the three Rho proteins exhibited a maximal activation around 2 hours in HUVEC intoxicated with 10<sup>-9</sup>M CNF1 (Fig. 1B). CNF1 interference with classical signaling pathways leading to gene regulation, has also been shown. Consistent with the absence of Ras activation measured, CNF1 did not produce ERK1/2 phosphorylation (Fig. 1A, 1C). CNF1 rather appeared to interfere both with the SAP-kinase signaling pathways, unraveled by p38MAP-kinase and cjun phosphorylations. CNF1 also interferes with the NF-kappaB pathway, as shown by IκB depletion (Fig. 1C). Host cells have evolved cell surface receptors to get

alarmed of the presence of PAMP (Medzhitov and Janeway, 2002). PAMP receptors initiate an innate immune response through I $\kappa$ B depletion for NF $\kappa$ B activation (Barton and Medzhitov, 2003). That cell treatment with the catalytic inactive CNF1-C866S toxin was devoid of interference with all signaling pathways tested, especially NF $\kappa$ B, strongly suggested an absence of cell recognition of CNF1 as a PAMP (Fig. 1C).

**Example 2 : Serum anti-OVA response following mucosal immunization of mice co-fed CNF1.**

Using a mouse model, characteristics of the host humoral response to CNF1 were investigated. Animals orally immunized with OVA, a prototype soluble antigen, co-administered with CNF1 (10  $\mu$ g) displayed serum IgG anti-OVA antibody responses (geometric mean titer 7768.7) comparable to those elicited by cholera toxin (geometric mean titer 6450) (Fig. 2). Under these experimental conditions, no serum anti-CNF1 responses were detected (not shown). It was also verified that neither CNF1 nor CT alone elicited production of seric anti-OVA IgG antibodies (not shown). Immunization with a lower dose of CNF1 (1  $\mu$ g) had negligible effects on serum anti-OVA responses when compared to control animals (geometric mean titers 868.7 and 787.5, respectively) (Fig. 2). Finally, immunization with 10  $\mu$ g of the catalytic inactive CNF1 mutant (CNF1-C866S) failed to enhance serum anti-OVA responses in animals co-fed OVA (Fig. 2). This result together with the fact that both CNF1 and CNF1-C866S were purified using identical conditions, excludes a possible stimulation of the IgG anti-OVA antibody responses by factors co-purified with CNF1. Collectively, these results show that the anti-OVA response elicited by CNF1 is dose dependent and requires its catalytic activity. As described for CNF1 (Doye et al., 2002), the catalytic domain of the closely related toxin DNT (DNT-CD) produced a transient activation of Rac due to the cellular depletion of this GTPase (Fig. 3A). Effects of DNT-CD were quantified using a classical HEp-2 cell assay, which gives a 50% multinucleation of cells at 10<sup>-12</sup>M CNF1 (Lemichez et al., 1997). In contrast to DNT-CD, which showed a 50% effect at 10<sup>-9</sup> M, the CNF1 catalytic domain CNF-CD had negligible effects (Fig. 3B). When immunostimulatory effects of both catalytic domains CNF-CD and



DNT-CD are compared, only mice immunized with 100 µg of DNT-CD developed a significant level of serum IgG anti-OVA antibodies (DNT-CD geometric mean titer of 7015 at 60 days) (Fig. 3C).

5    **Example 3 : Serum antibody isotype responses**

Sera from mice immunized with OVA together with 10 µg of CNF1, CNF1-C866S or CT were then tested for the presence of anti-OVA IgA and IgG subclasses. The isotype distribution of Ig anti-OVA antibody responses in animals immunized with CNF1 was similar to that observed  
10    in animals immunized with CT and was mainly accounted for by IgG1 and IgG2b. Likewise, mice fed a mixture of OVA and CNF1-C866S had no detectable anti-OVA antibody responses in any isotype (Fig. 4).

Taken together, these results indicate that CNF1, when given orally with OVA, promotes systemic anti-OVA responses with a profile of IgG  
15    subclasses similar to that induced by cholera toxin.

**Example 4 : Mucosal IgA antibody response**

Using the PERFEXT method, we then evaluated the ability of CNF1 to potentiate mucosal antibody responses in animals orally immunized with  
20    OVA. Sections of small intestine collected from groups of mice orally immunized with OVA, given together with CNF1 or CNF1-C866S, were analyzed for IgA content 2 weeks after the last of three immunizations. As illustrated in Fig. 5, oral co-administration of OVA with CNF1 elicited an antigen specific mucosal IgA response. Mice orally immunized with  
25    OVA given alone or admixed with the catalytic inactive CNF1-C866S had no detectable intestinal IgA antibody responses to OVA (Fig. 5).

**Example 5 : Histological analysis of CNF1 effects on small intestines**

30    Histological analyses of sections of small intestines prepared from mice immunized with CNF1 or CNF1-C866S showed no significant differences to those from control (bicarbonate fed) animals (Fig. 6).

**Example 6 : The catalytic domain of DNT remains active on cells and is sufficient to confer adjuvanticity**

35

CNF1 belongs to a family of toxins among them DNT, having similar catalytic activity (Boquet and Lemichez 2003). It is shown on Figure 3A that the catalytic domain of DNT (DNT-CTER) remains active on cells, although showing a lower intoxication property as compared to CNF1. Despite its inability to intoxicate cells (Fig. 7A), the catalytic domain of CNF1 (CNF1-CTER) upon mechanical injection into cells produces a *bona fide* toxic phenotype (Lemichez et al., 1997). It has been taken advantage of the above observations to test the adjuvant properties of the catalytic domains of both toxins. Mice were fed 10 times higher quantities of both toxin catalytic domains, as compared to CNF1. In these conditions it has been observed that DNT-CTER stimulated significantly the anti-OVA IgG responses (Fig. 7B). CNF1-CTER also produced a stimulation of the anti-OVA IgG responses, although at a lower level (Fig. 7B). Taken together, these results indicate that the adjuvanticity of this group of toxin is encompassed in their catalytic domain. Nevertheless, the injection domain of CNF1-toxin together with its catalytic domain, allows the use of lower doses to induce a significantly higher biological effect.

20

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